Replace the first full paragraph on page 11 (lines 2-14) with the following new paragraph rewritten in clean form:

The present invention provides a number of advantages. For example, as described in more detail below, the present antibody mimics exhibit improved biophysical properties, such as stability under reducing conditions and solubility at high concentrations. In addition, these molecules may be readily expressed and folded in prokaryotic systems, such as E. coli, in eukaryotic systems, such as yeast, and in in vitro translation systems, such as the rabbit reticulocyte lysate system. Moreover, these molecules are extremely amenable to affinity maturation techniques involving multiple cycles of selection, including in vitro selection using RNA-protein fusion technology (Roberts and Szostak, Proc. Natl. Acad. Sci USA 94:12297, 1997; Szostak et al., U.S.S.N. 09/007,005, now U.S. Patent No. 6,258,558 B1 and U.S.S.N. 09/247,190, now U.S. Patent No. 6,261,804 B1; Szostak et al. WO98/31700), phage display (see, for example, Smith and Petrenko, Chem. Rev. 97:317, 1997), and yeast display systems (see, for example, Boder and Wittrup, Nature Biotech. 15:553, 1997).

A2

Replace the third paragraph on page 12 (lines 7-11) with the following new paragraph rewritten in clean form:

FIGURE 5 is a photograph showing the structural similarities between a ^{10}Fn3 domain and 15 related proteins, including fibronectins, tenascins, collagens, and undulin. In this photograph, the regions are labeled as follows: constant, dark blue; conserved, light blue; neutral, white; variable, red; and RGD integrin-binding motif (variable), yellow.

A3

Replace the fourth paragraph on page 12 (lines 12-16) with the following new paragraph rewritten in clean form:

FIGURE 6 is a photograph showing space filling models of fibronectin III modules 9 and 10, in each of two different orientations. The two modules and the

A3

integrin binding loop (RGD) are labeled. In this figure, blue indicates positively charged residues, red indicates negatively charged residues, and white indicates uncharged residues.

Replace the first paragraph on page 15 (lines 2-17) with the following new paragraph rewritten in clean form:

A4

The antibody mimics of the present invention are based on the structure of a fibronectin module of type III (Fn3), a common domain found in mammalian blood and structural proteins. This domain occurs more than 400 times in the protein sequence database and has been estimated to occur in 2% of the proteins sequenced to date, including fibronectins, tenascin, intracellular cytoskeletal proteins, and prokaryotic enzymes (Bork and Doolittle, Proc. Natl. Acad. Sci. USA 89:8990, 1992; Bork et al., Nature Biotech. 15:553, 1997; Meinke et al., J. Bacteriol. 175:1910, 1993; Watanabe et al., J. Biol. Chem. 265:15659, 1990). In particular, these scaffolds include, as templates, the tenth module of human Fn3 (^{10}Fn3), which comprises 94 amino acid residues. The overall fold of this domain is closely related to that of the smallest functional antibody fragment, the variable region of the heavy chain, which comprises the entire antigen recognition unit in camel and llama IgG (Figure 1, 2). The major differences between camel and llama domains and the ^{10}Fn3 domain are that (i) ^{10}Fn3 has fewer beta strands (seven vs. nine) and (ii) the two beta sheets packed against each other are connected by a disulfide bridge in the camel and llama domains, but not in ^{10}Fn3.

Replace the paragraph beginning on page 20, line 19 and ending on page 21, line 14 with the following new paragraph rewritten in clean form:

A5

The antibody mimics described herein may be used in any technique for evolving new or improved binding proteins. In one particular example, the target of binding is immobilized on a solid support, such as a column resin or microtiter plate well, and the target contacted with a library of candidate scaffold-based binding proteins. Such a library may consist of ^{10}Fn3 clones constructed from the wild type ^{10}Fn3 scaffold through randomization of the sequence and/or the length of the ^{10}Fn3 CDR-like loops. If desired, this library may be an RNA-protein fusion library generated, for example, by the techniques described in Szostak et al., U.S.S.N. 09/007,005, now U.S. Patent No. 6,258,558 B1, and 09/247,190, now U.S. Patent No. 6,261,804 B1; Szostak et al., WO98/31700; and Roberts &

Szostak, Proc. Natl. Acad. Sci. USA (1997) vol. 94, p. 12297-12302. Alternatively, it may be a DNA-protein library (for example, as described in Lohse, DNA-Protein Fusions and Uses Thereof, U.S.S.N. 60/110,549, filed December 2, 1998, now abandoned, and 09/453,190, filed December 2, 1999). The fusion library is incubated with the immobilized target, the support is washed to remove non-specific binders, and the tightest binders are eluted under very stringent conditions and subjected to PCR to recover the sequence information or to create a new library of binders which may be used to repeat the selection process, with or without further mutagenesis of the sequence. A number of rounds of selection may be performed until binders of sufficient affinity for the antigen are obtained.

A5

Replace the paragraph beginning on page 22, line 22 and ending on page 23, line 11 with the following new paragraph rewritten in clean form:

A complex library was constructed from three DNA fragments, each of which contained one randomized area corresponding to a segment encoding a CDR-like loop. The fragments were named BC, DE, and FG, based on the names of the CDR-H-like loops encoded by them; in addition to encoding ^{10}Fn3 sequence and a randomized sequence, each of the fragments contained stretches encoding an N-terminal His_6 domain or a C-terminal FLAG peptide tag. At each junction between two fragments (i.e., between the BC and DE fragments or between the DE and FG fragments), each DNA fragment contained recognition sequences for the EarI Type IIS restriction endonuclease. This restriction enzyme allowed the splicing together of adjacent fragments while removing all foreign, non-^{10}Fn3-encoding, sequences. It also allows for a recombination-like mixing of the three ^{10}Fn3-encoding fragments between cycles of mutagenesis and selection.

A6

Replace the second paragraph on page 23 (lines 12-18), with the following new paragraph rewritten in clean form:

Each DNA fragment was assembled from two overlapping oligonucleotides, which were first annealed, then extended to form the double-stranded DNA form of the fragment. The oligonucleotides that were used to construct and process the three fragments are listed below; the "Top" and "Bottom" species for each fragment are the oligonucleotides that contained the

entire ^{10}Fn3 encoding sequence. In these oligonucleotides designations, "N" indicates A, T, C, or G; and "S" indicates C or G.

A6

Replace the header, on page 23, line 19, with the following header rewritten in clean form:

HFnLBCTop(His):

Replace the header, on page 24, line 1, with the following header rewritten in clean form:

A7

HfnLBCTop (an alternative N–terminus):

Replace the header, on page 25, line 15, with the following header rewritten in clean form:

A8

T7TMV (introduces T7 promoter and TMV untranslated region needed for in vitro translation):

Replace the first paragraph on page 26 (lines 2-4) with the following new paragraph rewritten in clean form:

A9

Unispl-s (splint oligonucleotide used to ligate mRNA to the puromycin-containing linker, described by Roberts et al, 1997, supra):
5'-TTTTTTTTTNAGCGGATGC-3' (SEQ ID NO: 13)

Replace the paragraph beginning on page 26, line 14 and ending on page 27, line 4 with the following new paragraph rewritten in clean form:

Next, each of the double-stranded DNA fragments was transformed into an RNA-protein fusion using the technique developed by Szostak et al., U.S.S.N. 09/007,005 now U.S. Patent No. 6,258,558 B1 and U.S.S.N. 09/247,190 now U.S. Patent No. 6,261,804 B1; Szostak et al., WO98/31700; and Roberts & Szostak, Proc. Natl. Acad. Sci. USA (1997) vol. 94, p. 12297-12302. Briefly, the fragments were transcribed using an Ambion in vitro transcription kit, MEGAshortscript (Ambion, Austin, TX), and the resulting mRNA was gel-purified and ligated to a DNA-puromycin linker using DNA ligase. The mRNA-DNA-puromycin molecule was then translated using the Ambion rabbit reticulocyte lysate-based translation kit. The resulting mRNA-DNA-puromycin-protein fusion was purified using Oligo(dT) cellulose, and a complementary DNA strand was synthesized using reverse transcriptase and the RT primers described above (Unisplint-S or flagASA), following the manufacturer's instructions.

A10 Replace the second paragraph on page 27 (lines 5-13) with the following new paragraph rewritten in clean form:

The RNA-protein fusion obtained for each fragment was next purified on the resin appropriate to its peptide purification tag, i.e., on Ni-NTA agarose for the His_6-tag and M2 agarose for the FLAG-tag, following the procedure recommended by the manufacturer. The cDNA component of the tag-binding RNA-protein fusions was amplified by PCR using Pharmacia Ready-to-Go PCR Beads, 10 pmol of 5' and 3' PCR primers, and the following PCR program (Pharmacia, Piscataway, NJ): Step 1: 95°C for 3 minutes; Step 2: 95°C for 30 seconds, 58/62°C for 30 seconds, 72°C for 1 minute, 20/25/30 cycles, as required; Step 3: 72°C for 5 minutes; Step 4: 4°C until end.

Replace the third paragraph on page 27 (lines 14-20) with the following new paragraph rewritten in clean form:

The resulting amplified DNA was cleaved by 5 U EarI (New England Biolabs) per1 ug DNA; the reaction took place in T4 DNA Ligase Buffer (New England Biolabs) at 37°C, for 1 hour, and was followed by an incubation at 70°C for15 minutes to inactivate Ear I. Equal amounts of the BC, DE, and FG DNA fragments were combined and ligated to form a full-length ^{10}Fn3 gene with

randomized loops. The ligation required 10 U of fresh EarI (New England Biolabs) and 20 U of T4 DNA Ligase (Promega, Madison, WI), and took 1 hour at 37°C.

Replace the paragraph starting on page 27, line 21 and ending on page 28, line 3 with the following new paragraph rewritten in clean form:

Three different DNA libraries were made in the manner described above. Each contained DNA encoding the form of the FG loop with 10 randomized residues. The DNA encoding the BC and the DE loops of the first library bore the wild type ^{10}Fn3 sequence; DNA encoding a BC loop with 7 randomized residues and a wild type DE loop made up the second library; and DNA encoding a BC loop with 7 randomized residues and a DE loop with 4 randomized residues made up the third library. The complexity of the DNA encoding the FG loop in each of these three libraries was 10^{13}; the further two randomized loops provided the potential for a complexity too large to be sampled in a laboratory.

a10

Replace the first paragraph on page 28 (lines 4-9) with the following new paragraph rewritten in clean form:

The three DNA libraries constructed were combined into one master library in order to simplify the selection process; target binding itself was expected to select the most suitable library for a particular challenge. RNA-protein fusions were obtained from the master DNA library following the general procedure described in Szostak et al., U.S.S.N. 09/007,005, now U.S. Patent No. 6,258,558 B1, and 09/247,190, now U.S. Patent No. 6,261,804 B1; Szostak et al., WO98/31700; and Roberts & Szostak, Proc. Natl. Acad. Sci. USA (1997) vol. 94, p. 12297-12302 (Figure 8).

Replace the second paragraph on page 28 (lines 11-19) with the following new paragraph rewritten in clean form:

The master library in the RNA-protein fusion form was subjected to selection for binding to TNF-α. Two protocols were employed: one in which the

a11

target was immobilized on an agarose column and one in which the target was immobilized on a BIACORE chip. First, an extensive optimization of conditions to minimize background binders to the agarose column yielded the favorable buffer conditions of 50 mM HEPES pH 7.4, 0.02% Triton, 100 μg/ml Sheared Salmon Sperm DNA. In this buffer, the non-specific binding of the ^{10}Fn3 RNA-protein fusion to TNF-α Sepharose was 0.3%. The non-specific binding background of the ^{10}Fn3 RNA-protein fusion to TNF-α Sepharose was found to be 0.1%.

Replace the third paragraph on page 28 (lines 20-24) with the following new paragraph rewritten in clean form:

A11

During each round of selection on TNF-α Sepharose, the RNA-protein fusion library was first preincubated for an hour with underivatized Sepharose to remove any remaining non-specific binders; the flow-through from this pre-clearing was incubated for another hour with TNF-α Sepharose. The TNF-α Sepharose was washed for 3-30 minutes.

Replace the first paragraph on page 29 (lines 1-5) with the following new paragraph rewritten in clean form:

After each selection, the cDNA from the RNA-protein fusion that had been eluted from the solid support with 0.3 M NaOH or 0.1M KOH was amplified by PCR; a DNA band of the expected size persisted through multiple rounds of selection (Figure 9); similar results were observed in the two alternative selection protocols, and only the data from the agarose column selection is shown in Figure 9.

Replace the second paragraph on page 29 (lines 6-10) with the following new paragraph rewritten in clean form:

In the first seven rounds, the binding of library RNA-protein fusions to the target remained low; in contrast, when free protein was translated from DNA pools at different stages of the selection, the proportion of the column binding

A11

species increased significantly between rounds (Figure 10). Similar selections may be carried out with any other binding species target (for example, IL-1 and IL-13).

Replace the third paragraph on page 29 (lines 12-19) with the following new paragraph rewritten in clean form:

A12

Wild-type ^{10}Fn3 contains an integrin-binding tripeptide motif, Arginine 78 - Glycine 79 - Aspartate 80 (the "RGD motif') at the tip of the FG loop. In order to avoid integrin binding and a potential inflammatory response based on this tripeptide in vivo, a mutant form of ^{10}Fn3 was generated that contained an inert sequence, Serine 78 - Glycine 79 - Glutamate 80 (the "SGE mutant"), a sequence which is found in the closely related, wild-type ^{11}Fn3 domain. This SGE mutant was expressed as an N-terminally His_6-tagged, free protein in E. coli, and purified to homogeneity on a metal chelate column followed by a size exclusion column.

Replace the paragraph beginning on page 36, line 23 and ending at page 37, line 9 with the following new paragraph rewritten in clean form:

A13

In one exemplary use for fibronectin scaffold selection on chips, a ^{10}Fn3-scaffold library-based selection was performed against TNF-α, using library of human ^{10}Fn3 variants with randomized loops BC, DE, and FG. The library was constructed from three DNA fragments, each of which contained nucleotide sequences that encoded approximately one third of human ^{10}Fn3, including one of the randomized loops. The DNA sequences that encoded the loop residues listed above were rebuilt by oligonucleotide synthesis, so that the codons for the residues of interest were replaced by (NNS)n, where N represents any of the four deoxyribonucleotides (A, C, G, or T), and S represents either C or G. The C-terminus of each fragment contained the sequence for the FLAG purification tag.

Replace the second paragraph on page 37 (lines 10-23) with the following new paragraph rewritten in clean form:

Once extended by Klenow, each DNA fragment was transcribed, and the transcript was ligated to a puromycin-containing DNA linker, and translated in vitro, as described by Szostak et al. (Roberts and Szostak, Proc. Natl. Acad. Sci USA 94:12297, 1997; Szostak et al., U.S.S.N. 09/007,005, now U.S. Patent No. 6,258,558 B1 and U.S.S.N. 09/247,190, now U.S. Patent No. 6,261,804 B1; Szostak et al., WO98/31700), to generate an mRNA-peptide fusion, which was then reverse-transcribed into a DNA-mRNA-peptide fusion. The binding of the FLAG-tagged peptide to M2 agarose separated full-length fusion molecules from those containing frameshifts or superfluous stop codons; the DNA associated with the purified full-length fusion was amplified by PCR, then the three DNA fragments were cut by Ear I restriction endonuclease and ligated to form the full length template. The template was transcribed, and the transcript was ligated to puromycin-containing DNA linkers, and translated to generate a ^{10}Fn3-RNA-protein fusion library, which was then reverse-transcribed to yield the DNA-mRNA-peptide fusion library which was subsequently used in the selection.

A13 Replace the third partial paragraph on page 37 (lines 24-25) with the following new partial paragraph rewritten in clean form:

Selection for TNF-α binders took place in 50 mM HEPES, pH 7.4, 0.02% Triton-X, 0.1 mg/mL salmon sperm DNA. The RNA-protein library was

Replace the first partial paragraph on page 38 (lines 1-4) with the following new partial paragraph rewritten in clean form:

incubated with Sepharose-immobilized TNF-α; after washing, the DNA associated with the tightest binders was eluted with 0.1 M KOH, amplified by PCR, and transcribed, and the transcript ligated, translated, and reverse-transcribed into the starting material for the next round of selection.

Replace the second paragraph on page 38 (lines 5-8) with the following new paragraph rewritten in clean form:

Ten rounds of such selection were performed (as shown in Figure 13); they

A15

resulted in an RNA-protein fusion pool that bound to TNF-α-Sepharose with the apparent average Kd of 120 nM. Specific clonal components of the pool that were characterized showed TNF-α binding in the range of 50-500 nM.

Replace the first partial paragraph on page 41 (lines 1-12) with the following new partial paragraph rewritten in clean form:

aminopropyltrimethoxysilane in 95% acetone / 5% water was prepared and allowed to hydrolyze for 20 minutes. The glass slides were immersed in the hydrolyzed silane solution for 5 minutes with gentle agitation. Excess silane was removed by subjecting the slides to ten 5-minute washes, using fresh portions of 95% acetone / 5% water for each wash, with gentle agitation. The slides were then cured by heating at 110°C for 20 minutes. The silane treated slides were immersed in a freshly prepared 0.2% solution of phenylene 1,4-diisothiocyanate in 90% DMF / 10% pyridine for two hours, with gentle agitation. The slides were washed sequentially with 90% DMF / 10% pyridine, methanol, and acetone. After air drying, the functionalized slides were stored at 0°C in a vacuum desiccator over anhydrous calcium sulfate. Similar results were obtained with commercial amine-reactive slides (3-D Link, Surmodics).

A14

Replace the second paragraph on page 41 (lines 13-24) with the following new paragraph rewritten in clean form:

Oligonucleotide capture probes were prepared with an automated DNA synthesizer (PE BioSystems Expedite 8909) using conventional phosphoramidite chemistry. All reagents were from Glen Research. Synthesis was initiated with a solid support bearing an orthogonally protected amino functionality, whereby the 3'-terminal amine is not unmasked until final deprotection step. The first four monomers to be added were hexaethylene oxide units, followed by the standard A, G, C and T monomers. All capture oligo sequences were cleaved from the solid support and deprotected with ammonium hydroxide, concentrated to dryness, precipitated in ethanol, and purified by reverse-phase HPLC using an acetonitrile gradient in triethylammonium acetate buffer. Appropriate fractions from the HPLC were collected, evaporated to dryness in a vacuum centrifuge, and then coevaporated with a portion of water.